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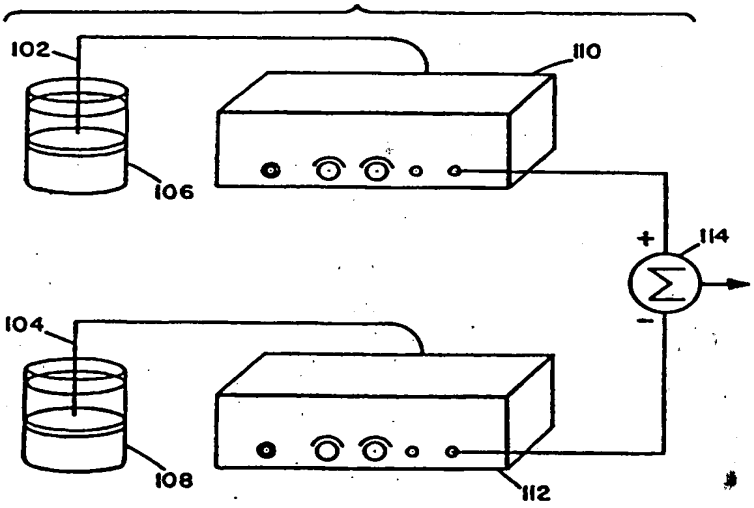

(57) Abstract

The detection system includes a pair f electrochemical hydrogen peroxide sensors, each sensor having working, counter and reference lectrodes. A bias voltage is applied to maintain a voltage difference between the working and reference electrodes. A sample aliquot of fluid was treated with either sodium azide or catalase. The sensors are placed in containers containing sufficient amounts of treated fluid to cover the active portions of the electrodes. The output current f each sensor is amplified, and the resulting amplified signals are combined and subtracted to provide a signal which is representative f the level f hydrogen peroxide in the fluid. In a method for assessing oxidative stress, including that related to essential hypertension, the detection system is used to determine a representative level of hydrogen peroxide in blood plasma drawn from a test subject. The level of hydrogen peroxide is directly related to the level of reactive oxygen species in the plasma, and can be used as an accurate predictor f risk for essential hypertension or other conditions related to oxidative stress.

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SYSTEM AND METHOD FOR MEASURING HYDROGEN PEROXIDE LEVELS IN A FLUID AND METHOD FOR ASSESSING OXIDATIVE STRESS

RELATED APPLICATIONS

This application claims the benefit of priority under 35 U.S.C.§119(e) to U.S. provisional application Serial No. 60/060,010 to Fred Lacy, Geert W. Schmid-Schoenbein, and David Gough, filed September 25, 1997, the subject matter of which is incorporated herein by reference.

FIELD OF THE INVENTION

The present invention relates to a hydrogen peroxide sensor for fluids, and applications therefore. In particular, the present invention relates to a sensor which permits continuous monitoring of hydrogen peroxide (H_2O_2) in fluids and use of the sensor in predicting essential hypertension and other oxidative stress-related conditions.

BACKGROUND

Hydrogen peroxide is formed in several biological and environmental processes. Hydrogen peroxide can be found in natural water (e.g., sea water, rain water), where it is an important species in redox reactions, in industrial processes, including drinking water purification, where it is used as a disinfectant, and in biological tissues, including blood, as a result of enzymatic reactions. Direct detection of hydrogen peroxide is an important analytical task, and numerous techniques have been devised for measurement of hydrogen peroxide levels in fluids as indications of, for example, medical conditions, environmental quality, or the presence of pathogens in cells of both animals and plants.

Superoxide radicals (O_2^-) in living tissue can be derived from many sources, such as activated granulocytes, endothelial cells, xanthine oxidase-catalyzed reactions, mitochondrial metabolism, and transition metal reactions with oxygen. Hydrogen peroxide (H_2O_2) can be produced from the dismutation of superoxide radicals catalyzed by the enzyme superoxide dimutase (SOD), from transition metal reactions with superoxide radicals, and from enzymes

(e.g.,glycollate oxidase and urate oxidase) which produce peroxide directly without first producing superoxide. The presence of antioxidants, certain enzymes such as SOD, and catalase serves to limit the concentration of the reactive oxygen species in plasma and tissues. Therefore, either an increase in the production of free radicals and/or a decrease in antioxidants can cause oxidative stress, contributing to possible cardiovascular complications in animals. Similarly, oxygen free radicals may affect vascular resistance by inactivating nitric oxide (NO), thereby causing arteriolar vasoconstriction and elevation of peripheral hemodynamic resistance. Other conditions have also been associated with oxidative stress, including acceleration of the progression of HIV to full-blown AIDS.

The mortality of individuals with hypertension has been found to be more than double that of the normotensive population, with most of the deaths occurring suddenly. Untreated hypertension also predisposes individuals to end organ damage or failure, including cerebrovascular accident (e.g., intracranial hemorrhage, encephalopathy), myocardial infarction, renal failure, and retinal hemorrhage. The mechanisms that predispose individuals with elevated arterial pressure to develop vascular organ injury are only partially understood. Oxygen free radicals and related intermediates have been implicated in hypertension and may play a role by affecting vascular smooth muscle contraction and resistance to blood flow. In individuals with histories of conditions such as atherosclerosis, stroke, and myocardial infarction, hypertension constitutes a risk factor.

Studies have shown that in persons with essential hypertension there exists not only reduced antioxidant enzyme and nitric oxide levels, but also an increase in the NADPH oxidase activity on neutrophil membranes. An increase in NADPH oxidase activity results in production of oxygen free radicals. Consequently, hypertensives (individuals experiencing increased systolic and diastolic blood pressures) have higher superoxide and hydrogen peroxide production by neutrophils than normotensive (individuals with normal blood pressure) controls. It has also been shown that hypertensive patients revert to normal free radical, antioxidant, and nitric oxide levels after effective antihypertensive treatment.

A number of different techniques are known for measurement of oxygen free radicals and their intermediates. These methods include the use of electrodes, chemiluminescence, and fluorescence. Specifically among techniques utilizing electrodes, a modification of the well known Clark electrode (see, e.g., Clark, L.C. Jr., *Trans. Am. Soc. Artif. Intern. Organs* 2, 41-48 (1956); Clark, L.C. Jr., *Ann. NY Acad. Sci.* 102, 29-45 (1962); Pat. No. 3,539,455 of Clark, Jr.) has been used to measure hydrogen peroxide levels from isolated neutrophils. All of the aforementioned methods are limited to measuring oxygen free radicals from stimulated neutrophils or deproteinized whole blood. As yet, there is no simplified clinical technique for measuring hydrogen peroxide in plasma.

SUMMARY OF THE INVENTION

An object of the present invention is to provide a novel detection system and method for determining hydrogen peroxide levels in a fluid.

Another object of the present invention is to provide a method for assessing essential hypertension by determining hydrogen peroxide levels in blood plasma.

Yet another objective of the present invention is to provide a method for measuring oxygen free radicals in blood plasma.

In an exemplary embodiment for measurement of hydrogen peroxide in blood plasma, the detection system includes a pair of electrochemical hydrogen peroxide sensors, each sensor having a platinum working electrode, a platinum counter electrode, and a silver/silver chloride reference electrode. A bias voltage is applied to maintain a voltage difference of 0.6V between the working and reference electrodes. The sensors are placed in containers containing sufficient amounts of treated blood samples to cover the active portions of the electrodes. The output current of each sensor is amplified, and the resulting amplified signals are combined and subtracted to provide a signal which is representative of the level of hydrogen peroxide in the plasma. In the first container, a sample aliquot of plasma was treated with sodium azide to stabilize the existing hydrogen peroxide by inhibition of blood catalase and

myeloperoxidase. In the second container, a second aliquot of the plasma was treated with catalase to deplete any hydrogen peroxide present in the sample in order to provide a baseline reading.

In a method for assessing oxidative stress, including that related to essential hypertension, the detection system is used to determine a representative level of hydrogen peroxide in blood plasma drawn from a test subject. The level of hydrogen peroxide is directly related to the level of reactive oxygen species in the plasma, and can be used as an accurate predictor of risk for essential hypertension.

Hydrogen peroxide levels in fluids other than blood may be measured using the previously-described system for measuring the differential in hydrogen peroxide between two identical samples of the fluid, one sample of which is treated to stabilize the hydrogen peroxide, the other sample of which is treated to deplete hydrogen peroxide.

BRIEF DESCRIPTION OF DRAWINGS

Understanding of the present invention will be facilitated by consideration of the following detailed description of a preferred embodiment of the present invention taken in conjunction with the accompanying drawings, in which like numerals refer to like parts and in which:

Figure 1 is a diagrammatic view of the hydrogen peroxide sensor system of the present invention;

Figures 2a, 2b and 2c are plots showing hydrogen peroxide levels in a PBS (phosphate buffer saline) solution with known concentrations of hydrogen peroxide and for two different plasma samples (each containing sodium azide) for electrochemical ($r=0.999$) (Figure 2a) and spectrophotometric ($r=0.998$) (Figure 2b) measurement techniques. Figure 2c compares the hydrogen peroxide levels in five plasma samples using the electrochemical and spectrophotometric techniques ($r=0.959$);

Figures 3a is a plot showing the stability of plasma hydrogen peroxide over time and Figure 3b is a bar graph comparing plasma hydrogen peroxide

levels in blood plasma obtained from centrifuged and sedimented samples, both measured using the spectrophotometric technique;

Figures 4a is a plot showing the correlation between measurements on fresh plasma hydrogen peroxide levels, using the electrochemical technique, and mean arterial blood pressure in hypertensive and normotensive human subjects, and Figure 4b is a bar graph showing the average plasma hydrogen peroxide levels of the three(3) groups; HTN, NTN,FH^+ (normotensive, family history of hypertension), and NTN,FH^-);

Figure 5 is a bar graph comparing hydrogen peroxide levels in blood plasma (measured with the electrochemical technique) after the addition of superoxide dimutase (SOD) and allopurinol;

Figures 6a and 6b are light micrographs of rat mesenteric microvessels after reaction tetranitroblue tetrazolium *in vivo*. Figure 6a show the arterioles and venules of high (6%) and low (0.3%) salt treated Dahl-R (salt resistant) animals; Figure 6b shows the arterioles and venules of high and low salt treated Dahl-S (salt sensitive) animals;

Figure 7 is a bar graph of light absorption measured along the endothelium of high (6%) and low (0.3%) salt treated Dahl-R and Dahl-S arterioles and venules;

Figure 8 is a bar graph of plasma hydrogen peroxide concentrations measured in high (6%) and low (0.3%) salt treated Dahl-R and Dahl-S animals using a Clark electrode; and

Figure 9 is a plot showing the correlation between plasma hydrogen peroxide concentrations versus mean arterial blood pressure measured in high (6%) and low (0.3%) salt treated Dahl-R and Dahl-S animals.

DETAILED DESCRIPTION OF PREFERRED EMBODIMENT

The following detailed description is addressed to the system and method for hydrogen peroxide detection as applied to blood. However, as will be readily apparent to those skilled in the art, the system and method described herein may be adapted for use in measurement of hydrogen peroxide in other

fluids, biological or not, and is not restricted to the exemplary embodiment that follows.

As shown in Figure 1, detection system 100 includes a pair of electrochemical hydrogen peroxide sensors 102, 104, each sensor having a platinum working electrode, a platinum counter electrode, and a silver/silver chloride reference electrode. A bias voltage is applied to maintain a voltage difference of 0.6V between the working and reference electrodes. The sensors 102,104 are placed in containers 106,108 containing sufficient amounts of treated blood samples to cover the active portions of electrodes 102,104. The output current of each sensor is amplified at amplifiers 110,112, and the resulting amplified signals are combined and subtracted at summer 114 to provide a signal which is representative of the level of hydrogen peroxide in the plasma. In first container 106, a sample aliquot of plasma was treated with sodium azide to stabilize the existing hydrogen peroxide by inhibition of blood catalase and myeloperoxidase. In second container 108, a second aliquot of the plasma was treated with catalase to deplete any hydrogen peroxide present in the sample in order to provide a baseline reading. The detection system was used in each case in which electrochemical measurements of hydrogen peroxide levels were taken.

The method of the present invention was initiated with the recruitment of patients with essential hypertension (with diastolic blood pressure (DBP) consistently >90 mmHg, or requiring antihypertensive treatment). Secondary hypertension was excluded by history, physical examination, and screening laboratory values (chemistry panel, hemogram, urinalysis, and electrocardiogram). Normotensive subjects (with a DBP consistently <85 mmHg) were recruited from institutional employees or from families of subjects with hypertension. Subjects were stratified by family history of hypertension, where a positive family history was defined as having a first degree relative with a documented DBP >90 mmHg (or requiring antihypertensive treatment) before the age of 60 years. Family history was ascertained by direct contact with the subjects parents (if alive). Table 1 summarizes the demographics of the clinical and biochemical characteristics of the subject groups including brachial arterial

systolic (SBP) and whether subjects were receiving any type of antihypertensive treatment, and, if so, what kind (e.g., angiotensin II antagonist or angiotensin converting enzyme inhibitor).

					Student t -test		2-way ANOVA		
	Normotensives (NT)			Hypertensive (HT)	HT vs NT	NT, (FH- vs FH+)	BP status (HT vs NT)	FH (FH- vs FH+)	Interaction (BP status x FH)
	FH-	FH+	all	all	P	P	P	P	P
n	14	15	29	21					
Demographic									
Age, years	45±4	38±4	42±3	56±2	**0.001**	0.19	**<0.001**	0.531	0.419
Race, W/B/other	12/1/1	8/1/6	20/2/7	14/5/2	χ^2=4.03, p=0.13	χ^2=4.34, p=0.11	0.133	0.099	0.09
Gender, M/F	10/4	6/9	16/13	16/5	χ^2=2.00, p=0.16	χ^2=2.89, p=0.089	0.082	0.115	0.963
CLINICAL									
BP medication (yes/no)	0/14	0/15	0/29	10/11	χ^2=14.4, **p<0.001**	—	**<0.001**	**0.015**	0.077
Height, cm	172±2	169±2	171±1	174±2	0.21	0.25	0.355	0.117	0.913
Weight, kg	79±4	80±5	80±3	90±3	**0.021**	0.76	0.081	0.484	0.482
BMI, kg/m^2	26.3±1.0	28.0±1.2	27.2±0.8	30.0±1.1	**0.046**	0.31	0.120	0.106	0.517
BSA, m^2	1.92±.05	1.90±.06	1.91±.04	2.04±.04	**0.026**	0.879	0.088	0.892	0.553
HR, beats/min	74±4	79±2	76±2	76±3	0.976	0.31	0.363	0.593	0.509
SBP, mmHg	115±2	124±3	119±2	146±4	**<0.001**	**0.022**	**<0.001**	**0.003**	**0.012**
MAP, mmHg	85±2	89±2	87±2	107±2	**<0.001**	0.18	**<0.001**	**0.001**	0.484
DBP, mmHg	70±3	72±2	71±2	87±2	**<0.001**	0.60	**<0.001**	**0.006**	0.396
BIOCHEMICAL									
Cortisol, mg/dL	15.1±1.1 (12)	13.5±1.4 (14)	14.2±0.9 (26)	14.3±3.9 (15)	0.958	0.41	0.886	0.122	0.782
Cholesterol, mg/dL	212±12 (12)	199±9 (11)	206±8 (23)	222±32 (15)	0.185	0.43	0.115	0.806	0.754
Triglycerides, mg/dL	156±25 (12)	152±25 (11)	154±18 (23)	186±88 (15)	0.276	0.91	0.598	0.747	0.659
Hematocrit, %	43.7±1.2	45.1±1.2	44.4±0.9	46.8±1.0	0.083	0.43	0.672	0.110	0.910
H_2O_2, μM	2.14±0.13	2.83±0.27	2.50±0.16	3.16±0.14	**0.005**	**0.03**	0.093	**0.003**	0.465

Mean ± one std. error.
Abbreviations: NT = normotensive; FH- = family history negative; FH+ = family history positive;; HT = hypertensive; BP = blood pressure; BMI = body mass index; BSA = body surface area; SBP = systolic blood pressure; MAP = mean arterial blood pressure; DBP = diastolic blood pressure; HR = heart rate.
Parenthesis indicate n, if different from total n for that column. P values <0.05 are in bold type.

TABLE 1

The following reagent stock solutions and blood plasma samples were prepared for use in determining plasma hydrogen peroxide levels:

A catalase stock solution was prepared by dissolving 25 mg of solid catalase into 2 ml of saline. The completed solution contained 250μg or 1,000 IU of catalase per 20μl of solution, which was capable of decomposing 10^{-3} moles of H_2O_2 at 30 C°. The catalase stock solution was stored at 0 C° for addition to the blood sample when appropriate.

A sodium azide stock solution was prepared by dissolving 650 mg of dry sodium azide into 5 ml of saline to create a solution with a final concentration of 2 mol/L. When added to a blood sample, 20μl of the stock solution gave a final sodium azide concentration of 50 mmol/L. The azide stock solution was stored at 0 C° for addition to the blood sample when appropriate.

A stock solution of phenol red was prepared by dissolving 50 mg of dry phenol red in 5 ml of distilled water. When added to a 10 ml blood sample, 10 μl of the 28 mmol/L phenol red stock solution had a final concentration of 2.8 mmol/L. The phenol red stock solution was stored at 0 C° until required for addition to blood samples.

A horseradish peroxidase stock solution was prepared by dissolving 27 mg (5000 U) of solid horseradish peroxidase into 5.4 ml of a 50 mmol/L potassium phosphate buffer, pH 7.0. The stock solution was stored at -70 C° in 0.5 ml aliquots. An amount of 10 μl (50 μg) of the horseradish peroxidase stock solution was added to each sample to be tested.

An SOD (superoxide dimutase) stock solution was prepared by dissolving 23 mg (75,000 U) of solid SOD into 2 ml of saline. A 20 μl portion of the SOD stock solution, which was stored at 0 C°, contained 0.23 mg (750 units) of SOD and was added to the blood sample being tested as needed. One unit of SOD is capable of inhibiting the rate of cytochrome c reduction by 50%.

The final stock solution, allopurinol, was obtained by dissolving 40 mg of dry allopurinol powder into 30 ml of saline solution to form a 10 mmol/L stock solution. Stored at 0 C°, 40 μl of the stock solution was added to the blood sample when appropriate.

The collection and preparation of the blood samples was initiated by the drawing of venous blood from an anticubital vein into a heparinized 10 ml Vacutainer™ tube. The anticoagulated sample was immediately set in ice until measurements were performed. For the samples, all measurements were completed within 4 hours of blood collection as previously performed pilot studies had shown that there were no detectable changes in the level of peroxide in a plasma sample over a 4 hour period. Samples were prepared for measurement by incubating 1.5 ml of blood in a 2 ml centrifuge tube at 37° C for 10 minutes and then centrifuging at 500 g for 10 minutes.

Sodium azide or catalase was added to the approximately 0.75 ml plasma layer that resulted from the incubation and centrifugation of the blood sample. Following the addition of sodium azide or catalase, the plasma was gently stirred such that the buffy coat and the red cell layers were not mixed with the higher plasma layer. The electrode was placed in the plasma (about 3 mm above the buffy coat) and its output was recorded for 10 minutes. After addition of catalase and sodium azide, measurements were performed in duplicate. The electrodes 102,104 were thoroughly cleaned between measurements, washing with a potent aqueous oxidizing solution (3% hydrogen peroxide) for one minute and then rinsing completely in a hydrogen peroxide free saline solution until the steady state baseline signal was re-established.

The hydrogen peroxide produced in a blood sample was measured with electrochemical sensor system 100 as shown in Figure 1. Each sensor comprises a platinum working electrode, a platinum counter electrode, and a silver/silver chloride reference electrode. A potential difference of 0.6 V was maintained between the working and reference electrodes. Hydrogen peroxide in the sample reacted at the working electrode surface to produce a current according to the reaction:

$$H_2O_2 - O_2 + 2\,H^+ + 2\,e^-$$

Using differential measurement according to the system shown in Figure 1, the current obtained from the plasma sample with catalase was subtracted

from the current obtained from the sample with sodium azide. The differences between these currents were attributed to the presence of hydrogen peroxide based upon the rationale that catalase causes the breakdown of hydrogen peroxide to oxygen and water according to the following reaction:

$$2\ H_2O_2 - O_2 + 2\ H_2O$$

The addition of catalase eliminates hydrogen peroxide and provides a baseline or background current. Sodium azide (NaN_3) inhibits the enzyme catalase and myeloperoxidase, which break down hydrogen peroxide, so that the sample with sodium azide gives the background current plus that which results from hydrogen peroxide. Subtracting the two currents provides a signal which is due to hydrogen peroxide. At the concentrations used in this procedure, neither catalase nor sodium azide alone yield a detectable electrode output current in saline solution.

The reproducibility of the hydrogen peroxide measurements according to the present invention were demonstrated by performing measurements of blood plasma with added sodium azide or catalase in duplicate. The measurements were reproducible and, on average, one measurement differed from another on the order of 3%.

Calibration of the sensor system was performed by adding known concentrations of hydrogen peroxide to a buffered saline solution, with and without sodium azide. A linear response for the current was found in the range between 0 and 10 μmol/L, as shown in Figure 2a. This same calibration procedure was performed on two plasma samples after addition of sodium azide which also yielded a linear calibration curve (Figure 2a). The output current was measured to three significant digits, and the calculated hydrogen peroxide concentration in the plasma samples had similar precision.

The electrochemical technique of the present invention was used to determine plasma hydrogen peroxide in five (5) samples before and after the addition of SOD, which facilitates superoxide dismutation to hydrogen peroxide, and allopurinol, which inhibits xanthine oxidase activity. A baseline current was

obtained from the sample after the addition of catalase. The hydrogen peroxide level was determined by subtracting the baseline electrode current from the current obtained after the addition of sodium azide. The hydrogen peroxide level was also documented after addition of superoxide dismutase together with sodium azide to plasma. Finally, after addition of allopurinol, superoxide dismutase, and sodium azide to the plasma sample, the hydrogen peroxide level was recorded.

To substantiate the values derived for plasma hydrogen peroxide from the novel electrode technique, hydrogen peroxide was measured in selected plasma samples using a conventional spectrophotometric technique based on horseradish peroxidase - mediated oxidation of phenol red by hydrogen peroxide. After the collection of a blood sample, a 500 μl aliquot was centrifuged at 500 g for 10 minutes. Of the resulting plasma, 100 μl was to each of two cuvettes. The amount of 10 μl of catalase was added to the first cuvette and sodium azide was added to the second. Horseradish peroxidase and phenol red were added to both cuvettes. The mixtures were allowed to react at room temperature for 10 minutes until the reactions were quenched with the addition of PBS (900 μl) and 1N sodium hydroxide (10 μl). The absorbance of oxidized phenol red at 610 nm was read for both cuvettes. The sample containing catalase served as the reference. The difference in light absorption of the two samples indicate the hydrogen peroxide concentration corroborating the levels measured using the inventive electrode technique, as can be seen by comparing Figures 2a and 2b. Absorbance readings using plasma diluted after the 10 minute reaction with horseradish peroxidase and phenol red gave reproducible readings of the oxidized phenol red.

A similar procedure was used on samples of blood prior to centrifugation to determine initial peroxide levels and the stability of these levels. Blood samples were collected as in previous sample collection. Within 10 seconds of phlebotomy, 600 μl of blood was placed into a syringe containing 30 μl of phenol red and 30 μl of horseradish peroxidase while another 600 μl was placed into a second syringe containing phenol red, horseradish peroxidase, and 60 μl of catalase. The two samples were centrifuged at 500 g for 10 minutes.

Following centrifugation, 100 μl of each of the two samples was placed into separate cuvettes to which 900 μl of PBS and 10 μl of sodium hydroxide were added. Again using conventional spectrophotometric techniques, the resulting absorbances were read at 610 nm. These readings represented the values at time t=0. The blood was kept on ice until subsequent measurements were taken at which point the sample was warmed at 37° C for 10 minutes after which the process was repeated.

Calibration for the spectrophotometric technique was performed by adding known concentrations of hydrogen peroxide to 10 μl sodium azide, horseradish peroxidase, phenol red, and 100 μl of PBS. After incubation for 10 minutes at room temperature, 900 μl of PBS and 10 μl of sodium hydroxide were added to stop the reaction. A linear absorbance response was obtained over the range of 0 to 10 μlmol/L (Figure 2b). The calibration procedure remained valid both in the presence and absence of sodium azide. The same calibration procedure was performed on two plasma samples after the addition of sodium azide where the plasma was a direct substitute for PBS of the original reaction.

Statistical measurements for the data resulting from blood sample testing were reported as the mean value +/- one standard error. Unpaired comparisons between mean values of the normotensive and hypertensive groups, or between the family history subgroups, were carried out by Student t-test. A 2-way ANOVA was used to simultaneously evaluate the effects of two independent variables upon plasma peroxide values. Paired nonparametric Wilcoxon signed rank tests were used to evaluate the effects of antihypertensive medications on hypertensive subjects, and to evaluate the effect of SOD and allopurinol on measured plasma peroxide (except for MAP in the antihypertensive medication study, paired t-tests gave the same results in terms of significant differences). Simple Pearson product-moment correlations were used to test relationships between variables. Differences between proportions of dichotomous variables were analyzed by χ^2 test. Multivariate analyses (stepwise multiple linear regression) were used to evaluate the impact of several independent demographic or clinical variables upon plasma peroxide levels as the dependent

variable. Analyses were performed using either Statworks and Cricket graph, or InStat. For either method of analyses, $p < 0.05$ was considered statistically significant.

During the calibration procedures, the electrochemical and spectrophotometric techniques gave linear responses (Figure 2a, b) when known amounts of hydrogen peroxide were added to PBS or plasma. When sodium azide was added to PBS, there was no change in the output of either measurement technique. However, the plasma samples that did not contain sodium azide produced a smaller response in comparison with PBS, while the addition of azide to the plasma samples resulted in the same response as the PBS counterpart. The intra-sample standard deviation in each case was less than that for the inter-sample standard deviation. Thus, it was determined that both techniques were suitable for measuring hydrogen peroxide when added to a plasma sample.

The precision of hydrogen peroxide measurements were calculated from the calibration curve. In the PBS and plasma samples with supplemented hydrogen peroxide concentrations, the average standard deviation was 0.188 nA for the electrode current and 0.011 for phenol red absorption. According to the calibration curves (Figures 2a,b), the standard deviation of the measured hydrogen peroxide level was 0.225 μlmol/L using the electrochemical technique and 0.183 μlmol/L using spectrophotometric technique. The purpose of the spectrophotometric technique was to independently affirm the plasma hydrogen peroxide values recorded by the electrochemical technique of the present invention. Plasma peroxide levels were found to be in the micromolar range using both the electrochemical and spectrophotometric techniques. When plasma peroxide concentrations were measured using both techniques a close correlation was observed ($r = 0.959$), as illustrated by Figure 2c.

For evaluation of the stability of endogenous hydrogen peroxide in blood, the same procedure as used in the spectrophotometric technique (reagents added to the blood sample prior to centrifugation) was utilized. The results of this evaluation are shown in Figure 3a. The plasma peroxide levels obtained immediately after the blood was drawn was the same as those obtained hours

after the blood was drawn. The plasma hydrogen peroxide levels remained constant over a 5 hour time period suggesting that the hydrogen peroxide in the blood samples was not generated *in vivo* or *ex vivo*, but instead already existed in the sample immediately after it had been drawn from the subject. Additional experiments were performed to compare the effects of sedimentation and centrifugation on plasma peroxide levels. Plasma peroxide levels obtained after centrifugation at 500 g were similar to those after sedimentation (1 g), as shown in the bar graph of Figure 3b, suggesting that the action of centrifugation does not constitute a source of hydrogen peroxide in the plasma samples.

Xanthine oxidase activity in plasma could represent a continuous source of reactive oxygen species. To determine the proportion of xanthine oxidase activity, the concentration of hydrogen peroxide was determined in five (5) plasma samples before and after the addition of SOD, to enhance superoxide dismutation to hydrogen peroxide and allopurinol, to block xanthine oxidase activity. Referring to Figure 5, it can be seen that upon addition of superoxide dismutase (SOD) and sodium azide to the plasma, the measured hydrogen peroxide increased by 38%. In contrast, upon the addition of allopurinol, SOD, and sodium azide to plasma, a 60% reduction in hydrogen peroxide level was observed in comparison with the hydrogen peroxide levels seen in plasma containing only sodium azide. Further, there was a 71% reduction of measured hydrogen peroxide compared to the levels seen in plasma that contained only SOD and sodium azide.

Referring back to Table 1, when comparing the demographic, clinical and biochemical characteristics of the test subject groups, subjects in the hypertensive group had substantially elevated systolic, diastolic, and mean arterial blood pressures ($p < 0.001$) than those in the normotensive subject group. Corresponding to the 23% (20 mmHg) elevation in mean arterial pressure, the average hydrogen peroxide concentration showed an increase of 26% (0.66 μmol/L; $p = 0.005$) in the hypertensive group when compared to the normotensive group.

When the normotensive group was stratified by family history of hypertension, family history positive normotensives had higher hydrogen

peroxide levels by 32% (0.69 μmol/L; $p = 0.03$) than those subjects with a negative family history of hypertension. Further, the family history positive normotensives had higher systolic blood pressures by 8% (9 mmHg), though diastolic and mean arterial blood pressures remained similar to the family history negative normotensives. When hypertensives were compared with family history negative normotensives (*i.e.*, comparing hypertensive with true controls), it was found that the hypertensive group had a higher mean arterial pressure by 23% (20 mmHg) as well as higher levels of plasma hydrogen peroxide by 48% (1.02 μmol/L) over the normotensive control.

Comparison of the data from the hypertensive group and the normotensive group shows a linear correlation between blood pressure and plasma hydrogen peroxide of $r = 0.54$ ($n = 50$; $p < 0.001$). However, when the hypertensive group was compared only with normotensives who had no family history of hypertension, the correlation improved ($r = 0.70$; $n = 35$; $p < 0.001$), as shown in the plot of Figure 4a as well as in Table 2, provided below. The plasma hydrogen peroxide concentration of subjects in the normotensive with a positive family history of hypertension group fell between the levels for those in the hypertensive group and normotensive negative family history group, as shown in the bar graph provided in Figure 4b.

Independent variable	Coefficient	Standard error of the estimate	t statistic	p value
Family history of Hypertension	-4.87	1.83	-2.66	0.012
Age	0.10	0.07	1.40	0.170
Race	1.98	2.35	0.85	0.403
Gender	-1.01	1.88	-0.53	0.597

Multiple Linear regression statics for plasma hydrogen peroxide as a function of demographic characteristics, as well as family history of hypertension. This model predicts plasma H_2O_2 with $F = 3.34$, $p = 0.022$, $r = 0.55$, $r^2 = 0.30$, $n = 46$.

TABLE 2

Because 10 of the 21 subjects in the hypertensive group were taking antihypertensive medications at the time of plasma sampling for hydrogen peroxide, the subjects were stratified by presence (n = 10) or absence (n = 11) of treatment. However, treatment showed to have no effect on hydrogen peroxide levels in the unpaired sample (t = -1.62, p = 0.12). The effects of antihypertensive treatment (with an angiotensin II receptor antagonist or an angiotensin converting enzyme inhibitor) versus control was evaluated in five (5) of the essential hypertensive subjects. This information is provided in Table 3.

	Untreated	Treated	p (Wilcoxon paired)
Clinical			
Weight, kg	93.2 ± 5.5	92.9 ± 5.5	0.250
BMI, kg/m_2	30.4 ± 1.8	30.2 ± 1.8	0.250
BSA, M^2	2.09 ± 0.05	2.08 ± 0.05	0.250
HR, beats/min	79 ± 6	74 ± 3	0.094
SBP, mmHg	163 ± 9	146 ± 11	0.031
MAP, mmHg	111 ± 4	104 ± 5	0.031
DBP, mmHg	85 ± 4	83 ± 4	0.219
Biochemical			
Cortisol, μg/dl	13.7 ± 2.1	13.7 ± 1.6	0.500
Cholesterol, mg/dl	236.6 ± 8.7	238.6 ± 8.5	0.406
Triglycerides, mg/dl	149.8 ± 16.3	138.4 ± 23.5	0.312
Hematocrit, %	46.1 ± 1.5	44.7 ± 1.6	0.312
H_2O_2, μM	3.11 ± 0.27	2.50 ± 0.29	0.031

N = 5 uncomplicated essential hypertensive subjects studied both off medications (for 3 days), and after treatment with either the angiotensin II receptor antagonist eprosartan (n = 4; 100-300 mg orally twice daily) or the angiotensin converting enzyme antagonist enalapril (n = 1; 10 mg orally twice daily). Results are mean ± one standard error. Abbreviations: defined in Table 1 legend.

TABLE 3

In this selected group, treatment, causing the disruption of the renin-angiotensin system, lowered plasma hydrogen peroxide by 20% (0.61 μmol/L, p = 0.018) as well as systolic pressure by 10% (17 mmHg, p = 0.026).

Because it was shown that both blood pressure status (*i.e.*, labeling a person as hypertensive or normotensive) and hypertension family history influenced plasma hydrogen peroxide (Table 3), a 2-way ANOVA was undertaken. The results showed that family history was of greater importance than blood pressure status for determining a patient's plasma peroxide concentration ($p = 0.003$ versus $p = 0.093$).

The importance of age, race, and gender, along with a family history of hypertension as possible determinants of plasma hydrogen peroxide were evaluated by multiple linear regression. The results, as shown in Table 2, reveal that, once again, family history was the best predictor of plasma hydrogen peroxide ($t = -2.66$, $p = 0.012$), while the other demographic variables were of little significance (all $p > 0.1$).

While subjects in the normotensive and hypertensive groups did not differ in plasma biochemistry other than the level of hydrogen peroxide (Table 1), members of the hypertensive group measured, on the average, larger in weight, body mass index, and body surface area. These variables were evaluated by multiple linear regression, along with those subjects from the hypertension family history group, as possible determinants of plasma hydrogen peroxide. Once again, as shown in Table 4, family history remained the best predictor ($t = -3.19$, $p = 0.003$). Body surface area ($t = 1.79$, $p = 0.08$) and body weight ($t = -1.78$, $p = 0.081$) were marginal predictors, while neither height or body mass index showed a relationship to plasma hydrogen peroxide concentration ($p > 0.2$).

Independent variable	Coefficient	Standard error of the estimate	t statistic	p value
Family history of Hypertension	-5.32	1.67	-3.19	0.003
Body surface area	0.02	0.01	1.79	0.080
Weight	-2.57	1.44	-1.78	0.081
Height	-143.50	115.60	-1.24	0.221
Body mass index	1.21	1.45	0.83	0.409

Multiple linear regression statistics for plasma hydrogen peroxide as a function of body size characteristics (clinical parameters), as well as family history of hypertension. This model predicts plasma H_2O_2 with $F = 4.78$, $p = 0.002$, $r = 0.61$, $r^2 = 0.037$, $n = 46$.

TABLE 4

The following example provides an illustration of an application of the system and method of the present invention to evaluation of hypertension in rats.

Example 1:

There is evidence that oxygen free radicals are involved in the development of salt induced hypertension. Dahl salt-sensitive (Dahl-S) and Dahl salt-resistant (Dahl-R) rats were divided into four groups. One group of the Dahl-S and one group of the Dahl-R rats were fed a high (6.0% NaCl) salt diet for four weeks. The other two groups were fed a low (0.3% NaCl) salt diet for the same period. It was established that a high salt diet resulted in severe hypertension in the Dahl-S subjects but had no effect on blood pressure in the Dahl-R animals. Along with the use of tetranitoblue tetrazolium dye to detect superoxide radicals in microvessels of the mesentery, the apparatus and method of the present invention was used to measure hydrogen peroxide levels in fresh plasma taken from the animals.

Blood samples were taken from the femoral arterial catheter of the Dahl-S and Dahl-R rats using heparin as an anticoagulant. The samples were immediately placed on ice until all the measurements were performed. All measurements were completed on the samples within 2-3 hours, a period of time that did not significantly affect the peroxide levels in these samples.

To measure hydrogen peroxide levels, a 750 μl aliquot of anticoagulated blood was placed in a 2 ml centrifuge tube, incubated at 37° C for ten minutes, and then centrifuged at 500 g for 10 minutes. After centrifugation, either sodium azide or catalase was added to the resulting plasma layer and gently stirred. An electrode was placed in the sample plasma (approx. 3 mm above the buffy coat) and its output was recorded for 10 minutes, at which point a steady electrode current was reached. Measurements were performed in duplicate and the electrode was cleaned between measurements by a potent aqueous oxidizing solution (3% hydrogen peroxide).

Because the output voltage generated by the electrode was not specific for hydrogen peroxide, differential measurement were carried out using samples containing sodium azide and sodium azide with catalase. The enzyme catalase serves to eliminate hydrogen peroxide by breaking it down into the

component parts, water and oxygen. Thus, the addition of catalase to the plasma sample provides a baseline or background current for sample testing. In contrast, sodium azide inhibits the action of catalase therefore the electrochemical value for a sample containing azide gives the current due to hydrogen peroxide as well as any background. The difference between these two currents provides a signal which is due solely to the hydrogen peroxide in the sample.

Hypertensive, salt treated Dahl-S animals showed a significantly higher plasma hydrogen peroxide concentration when compared to its normotensive, low salt control (2.81∓0.43 versus 2.10∓0.41 μmol/L; $p<0.01$; Figure 8). In contrast, no difference in plasma peroxide was detected between the high and low salt treated Dahl-R animals (1.70∓0.35 versus 1.56∓0.51 μmol/L). Further, both groups of Dahl-R animals had lower plasma peroxide values than either group of Dahl-S animals. As shown in Figure 9, plasma hydrogen peroxide concentrations showed a linear correlation with mean arterial blood pressure in all four (4) experimental groups ($r=0.77$, $n=35$).

Other tests were performed on the rats to confirm the relationship between the hydrogen peroxide measurement method of detecting hypertension and the presence of essential hypertension, including intravital microscopy on mesenteric microvessels. Figures 6a and 6b illustrate a set of light micrographs of rat mesenteric microvessels after reaction tetranitroblue tetrazolium *in vivo*. Figure 6a, consisting of four images, depicts the arterioles and venules of high (6%) and low (0.3%) salt treated Dahl-R (salt resistant) animals. Figure 6b, also four images, depicts the arterioles and venules of high and low salt treated Dahl-S (salt sensitive) animals, showing increased staining in the high salt treated Dahl-S arterioles and venules. Figure 7 is a histogram showing the levels of light absorption measured along the endothelium of high (6%) and low (0.3%) salt treated Dahl-R and Dahl-S arterioles and venules. The clear bars on the graph represent the values for low salt treatment while the shaded bars represent the values for high salt treatment.

The above-described system and method for measurement of hydrogen peroxide levels in fluid provides means for accurately assessing the condition of

essential hypertension in a subject when used on blood plasma samples from the subject. The method can be performed quickly with relatively simple and inexpensive equipment. In fact, the equipment set-up is so simple, that it is conceivable that personal test systems could be made available to patients for regular monitoring of a pre-diagnosed condition. As additional relationships between oxidative stress and other medical conditions are determined, the inventive method may be used for screening and monitoring for such conditions. The system and method are not limited only to evaluation of hydrogen peroxide in blood, but may be applied to measurement of hydrogen peroxide in other fluids

It will be apparent to those skilled in the art that various modifications and variations may be made in the system and method of the present invention without departing from the spirit or scope of the invention. Thus, it is intended that the present invention cover the modification and variations of this invention provided they come within the scope of the appended claims and their equivalents.

We claim:

CLAIMS

1. A system for measurement of oxidative stress in a subject, the system comprising:

a first mixture contained in a first container, the first mixture comprising a first aliquot of a blood sample taken from the subject and a stabilizer for inhibiting blood catalase and myeloperoxidase;

a second mixture contained in a second container, the second mixture comprising a second aliquot of the blood sample taken from the subject and a catalase for depleting any hydrogen peroxide within the blood sample;

a first sensor disposed within the first container for detecting a first electrical current within the first mixture;

a second sensor disposed within the second container for detecting a second electrical current within the second mixture; and

means for combining and differentiating the first and second electrical currents and for generating a difference signal therefrom;

wherein the difference signal corresponds to a level of hydrogen peroxide in the subject's blood, and the level of hydrogen peroxide corresponds to oxidative stress.

2. The system of claim 1, wherein each of the first and second sensors comprises a working electrode, a counter electrode and a reference electrode.

3. The system of claim 2, wherein a bias voltage is applied to each of the first and second sensors to maintain a voltage difference between the working electrode and the reference electrode.

4. The system of claim 2, wherein the working electrode and the counter electrode are formed from platinum.

5. The system of claim 2, wherein the reference electrode is silver/silver chloride.

6. The system of claim 1, wherein the stabilizer is sodium azide.

7. The system of claim 1, wherein the blood sample is centrifuged prior to creation of the first and second mixtures to provide a separated blood sample having a substantially clear top layer of blood plasma.

8. A method for measuring hydrogen peroxide in a blood sample, the method comprising:

separating a first and a second aliquot of the blood sample into at least a bottom layer and a top layer, wherein the top layer is a plasma;

incubating the plasma of the first aliquot with a first compound capable of degrading hydrogen peroxide;

incubating the plasma of the second aliquot with a second compound capable of inhibiting activity of an enzyme known to degrade hydrogen peroxide;

disposing an electrochemical sensor from a sensor system in each of the first and second aliquots and generating a first and second current signal;

determining a difference between the first and second current signals, wherein the difference corresponds to a concentration of hydrogen peroxide.

9. The method of claim 8, wherein the first compound is sodium azide.

10. The method of claim 8, wherein the second compound is catalase, and the catalase is in stoichiometric excess.

11. The method of claim 8, further comprising calibrating the sensor system by placing the electrochemical sensors in known concentrations of hydrogen peroxide in a buffered saline solution to generate a calibration curve.

12. A method for assessing essential hypertension, the method comprising:

centrifuging a first and a second aliquot of the blood sample to separate each aliquot into at least a bottom layer and a top layer, wherein the top layer is a plasma;

treating the plasma of the first aliquot with a sodium azide;

treating the plasma of the second aliquot with catalase;

obtaining an amperometric measurement from the plasma in each of the first and second aliquots to generate a first and a second current signal;

determining a difference between the first and second current signals, wherein the difference corresponds to a concentration of hydrogen peroxide;

comparing the concentration of hydrogen peroxide to a mean hydrogen peroxide level of an appropriate demographic group in order to determine whether the concentration of hydrogen period indicates a pathogenesis associated with essential hypertension.

13. The method of claim 12, wherein the blood sample is anticoagulated.

14. The method of claim 12, wherein the catalase is in stoichiometric excess.

15. The method of claim 12, further comprising calibrating the sensor system by placing the electrochemical sensors in known concentrations of hydrogen peroxide in a buffered saline solution to generate a calibration curve.

16. The method of claim 12, wherein the demographic group is one of a normotensive with a family history of hypertension and a normotensive without a family history of hypertension.

17. A method for determining a level of oxidative stress in a subject, the method comprising:

obtaining a first and a second aliquot of a blood plasma from the subject;

incubating the blood plasma of the first aliquot with a first compound capable of degrading hydrogen peroxide;

incubating the plasma of the second aliquot with a second compound capable of inhibiting activity of an enzyme known to degrade hydrogen peroxide;

disposing an electrochemical sensor from a sensor system in each of the first and second aliquots and generating a first and second current signal; and

determining a difference between the first and second current signals, wherein the difference corresponds to a concentration of hydrogen peroxide;

wherein the concentration of hydrogen peroxide corresponds to a level of oxygen free radicals in the blood plasma.

18. The method of claim 17, wherein the first compound is sodium azide.

19. The method of claim 17, wherein the second compound is catalase, and the catalase is in stoichiometric excess.

20. The method of claim 17, further comprising calibrating the sensor system by placing the electrochemical sensors in known concentrations of hydrogen peroxide in a buffered saline solution to generate a calibration curve.

21. A system for measurement of hydrogen peroxide within a fluid, the system comprising:

a first mixture contained in a first container, the first mixture comprising a first sample of the fluid and a stabilizer for inhibiting breakdown of hydrogen peroxide within the fluid;

a second mixture contained in a second container, the second mixture comprising a second sample of the fluid and a catalase for depleting any hydrogen peroxide within the second sample;

a first sensor disposed within the first container for detecting a first electrical current within the first mixture;

a second sensor disposed within the second container for detecting a second electrical current within the second mixture; and

means for combining and differentiating the first and second electrical currents and for generating a difference signal therefrom;

wherein the difference signal corresponds to a level of hydrogen peroxide in fluid.

22. The system of claim 21, wherein each of the first and second sensors comprises a working electrode, a counter electrode and a reference electrode.

23. The system of claim 22, wherein a bias voltage is applied to each of the first and second sensors to maintain a voltage difference between the working electrode and the reference electrode.

24. The system of claim 22, wherein the working electrode and the counter electrode are formed from platinum.

25. The system of claim 22, wherein the reference electrode is silver/silver chloride.

26. The system of claim 21, wherein the stabilizer is sodium azide.

27. A method for measuring hydrogen peroxide in a fluid sample, the method comprising:

dividing the fluid sample into at least a first sample and a second sample;

incubating the first sample with a first compound capable of degrading hydrogen peroxide;

incubating the second sample with a second compound capable of inhibiting activity of an enzyme known to degrade hydrogen peroxide;

disposing an electrochemical sensor from a sensor system in each of the first and second samples and generating a first and second current signal;

determining a difference between the first and second current signals, wherein the difference corresponds to a concentration of hydrogen peroxide.

28. The method of claim 27, wherein the first compound is sodium azide.

29. The method of claim 27, wherein the second compound is catalase, and the catalase is in stoichiometric excess.

30. The method of claim 27, further comprising calibrating the sensor system by placing the electrochemical sensors in known concentrations of hydrogen peroxide in a buffered saline solution to generate a calibration curve.

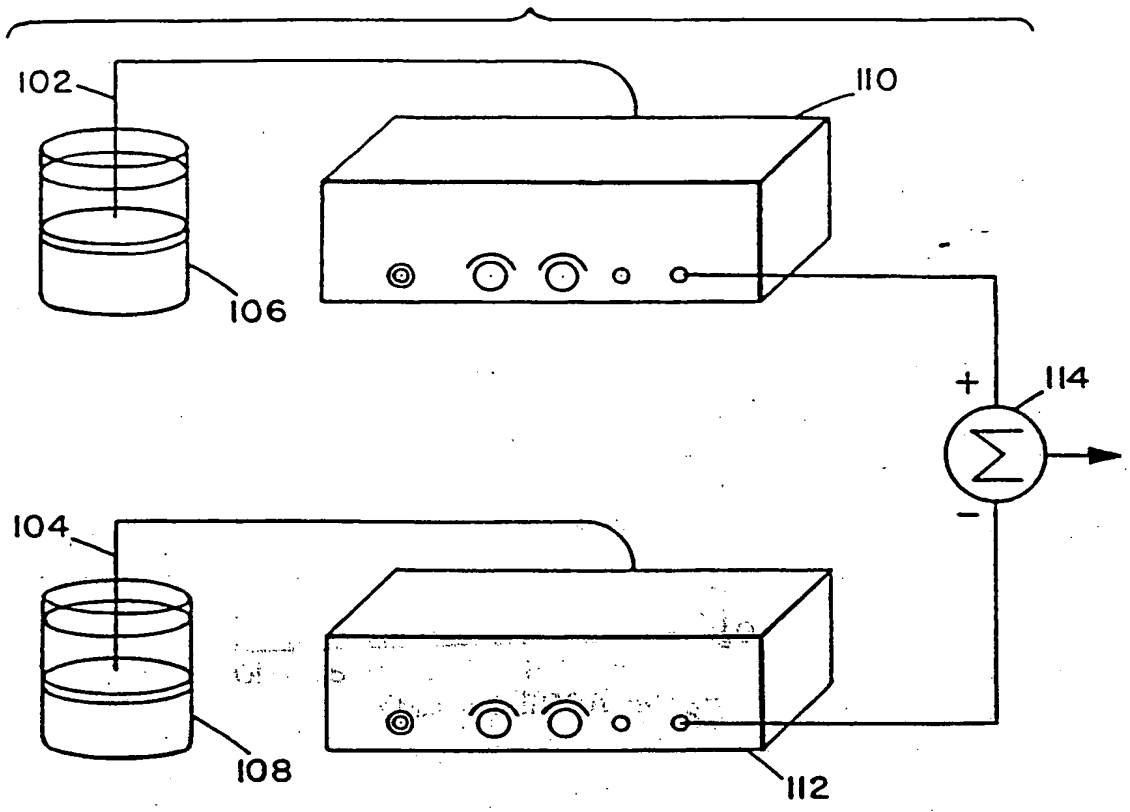
102
110
106
+
114
Σ
−
104
108
112

FIG. 1

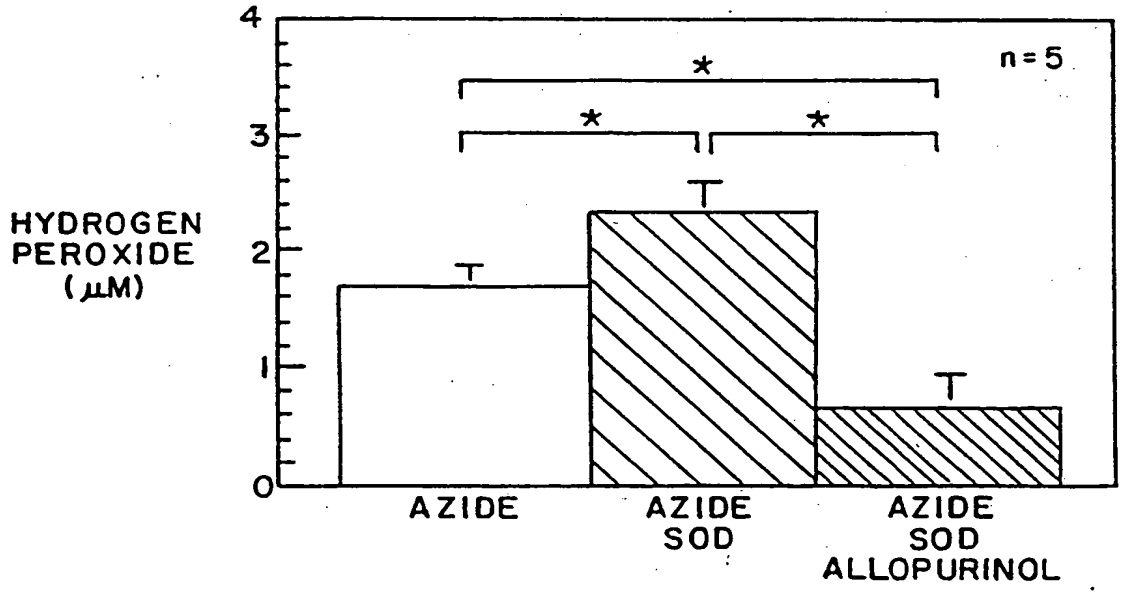
HYDROGEN PEROXIDE (μM)
4
3
2
1
0
n = 5
*
*
*
AZIDE
AZIDE SOD
AZIDE SOD ALLOPURINOL

FIG. 5

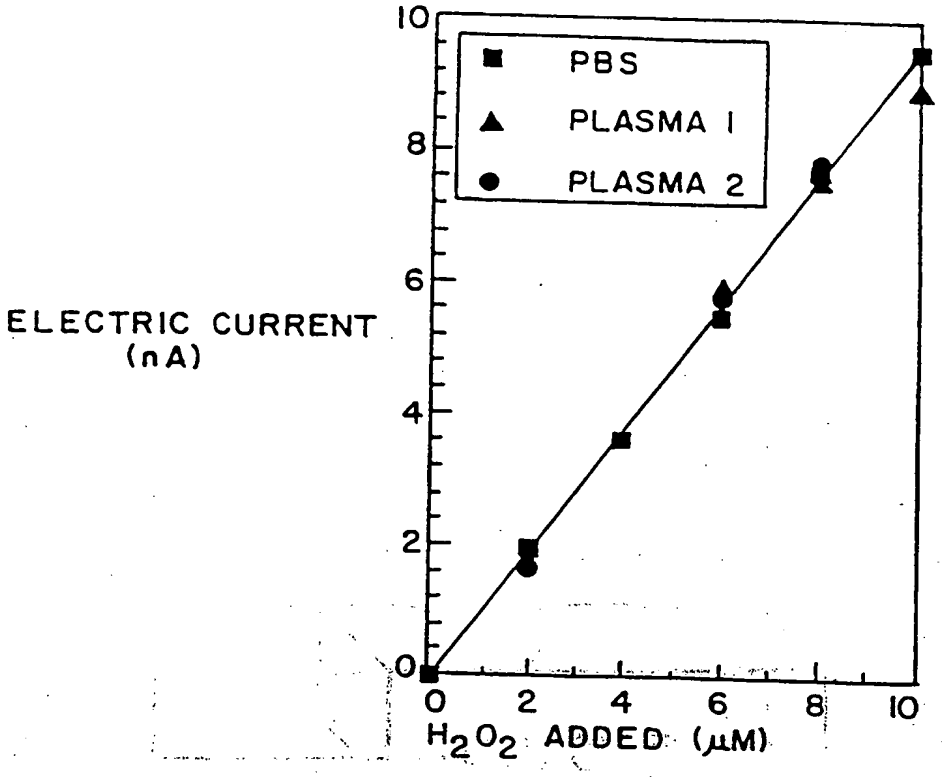

FIG. 2a

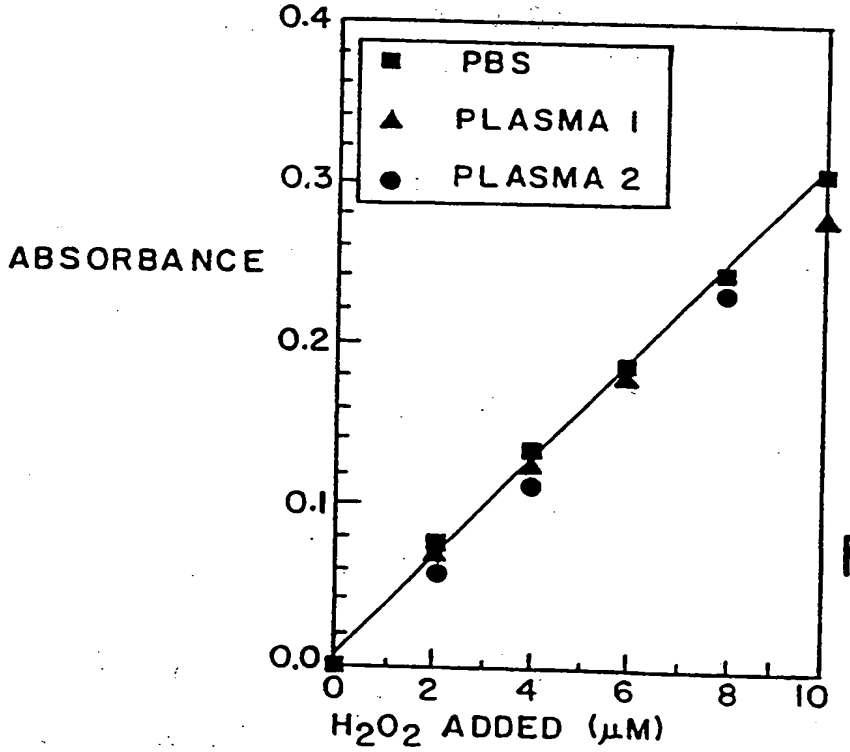

FIG. 2b

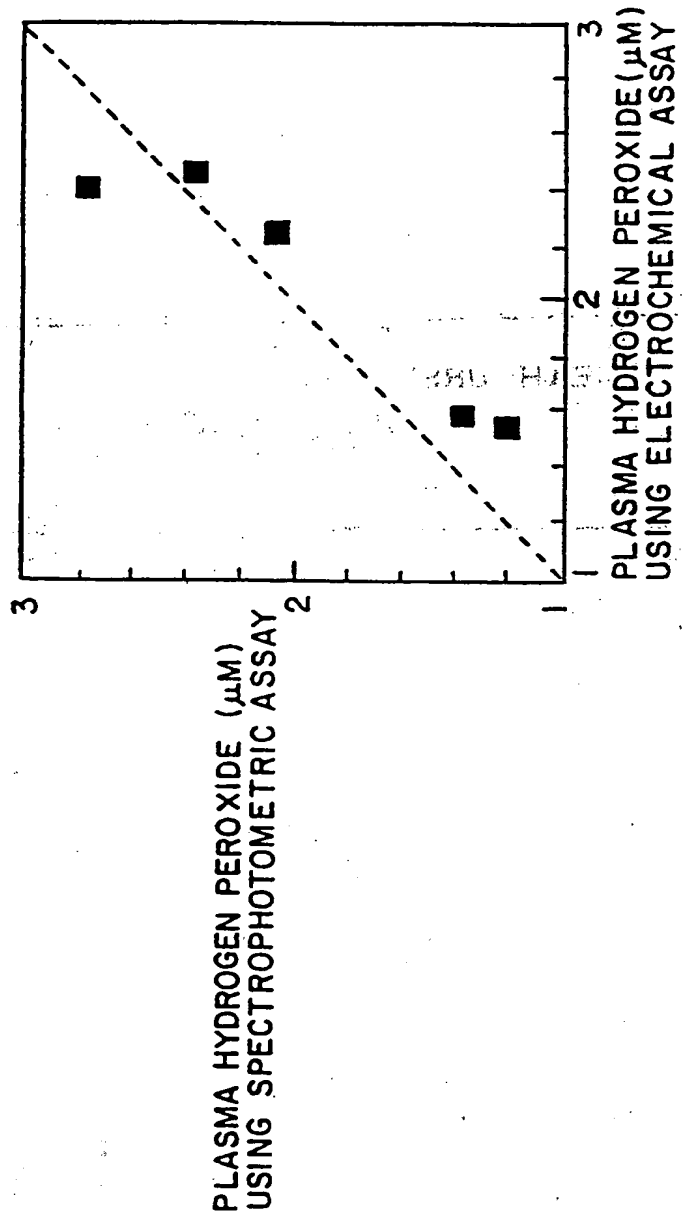
PLASMA HYDROGEN PEROXIDE (μM)
USING SPECTROPHOTOMETRIC ASSAY
PLASMA HYDROGEN PEROXIDE(μM)
USING ELECTROCHEMICAL ASSAY
1
2
3
1
2
3

FIG. 2C

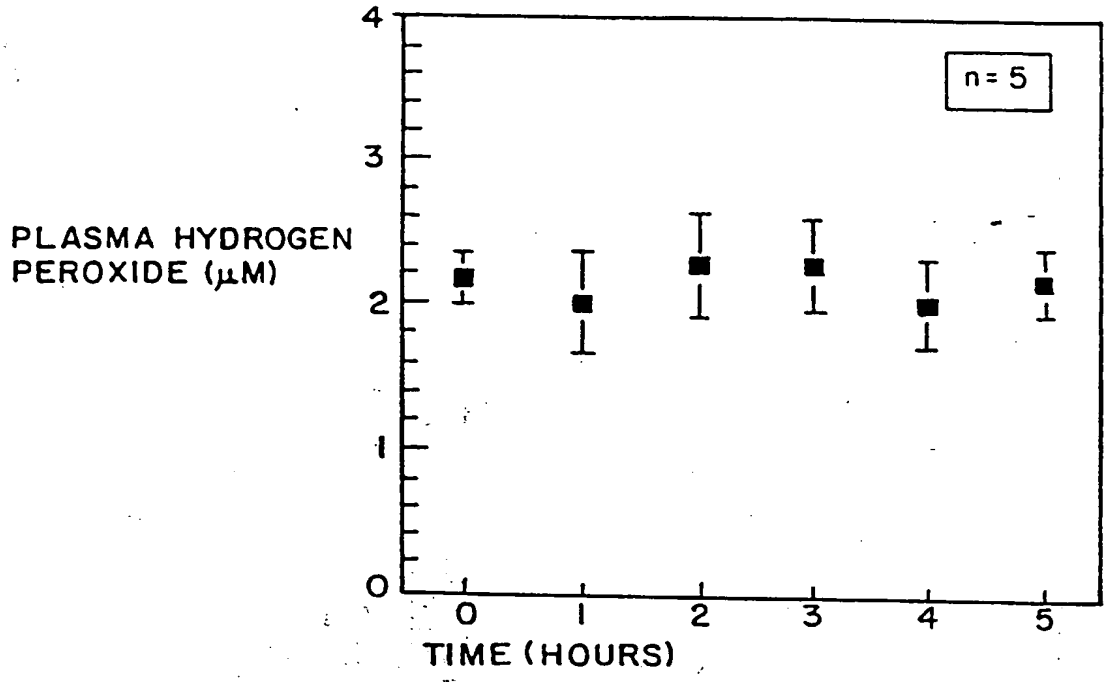
PLASMA HYDROGEN PEROXIDE (μM)
n = 5
4
3
2
1
0
0
1
2
3
4
5
TIME (HOURS)

FIG. 3a

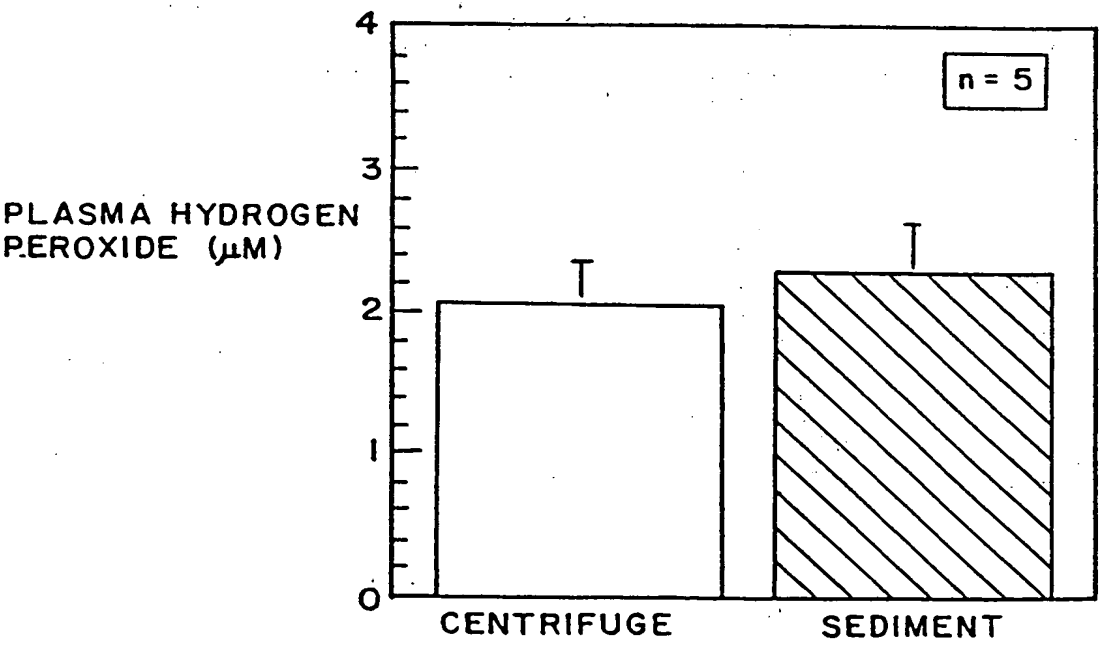
PLASMA HYDROGEN PEROXIDE (μM)
n = 5
4
3
2
1
0
CENTRIFUGE
SEDIMENT

FIG. 3b

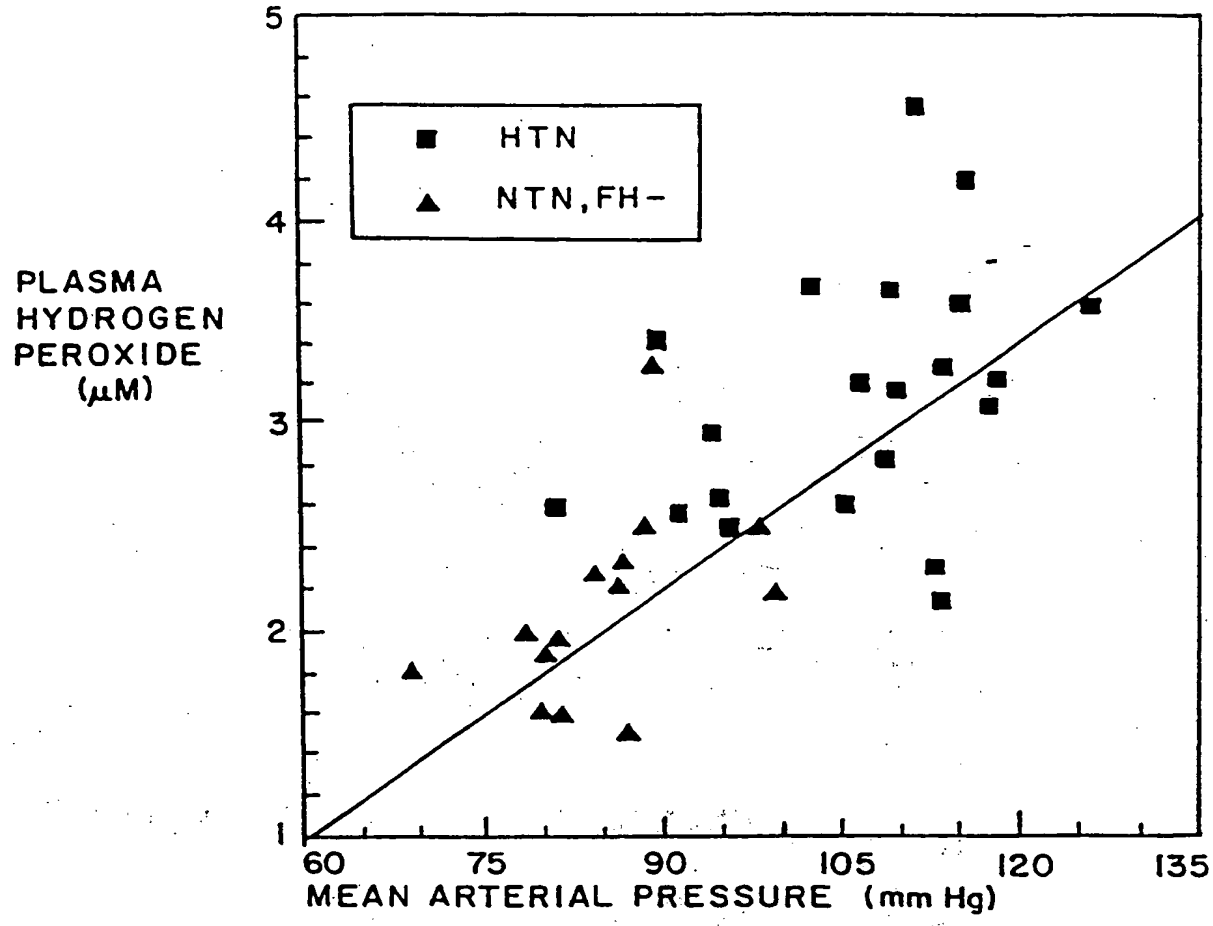
PLASMA HYDROGEN PEROXIDE (μM)
HTN
NTN, FH−
5
4
3
2
1
60
75
90
105
120
135
MEAN ARTERIAL PRESSURE (mm Hg)

FIG. 4a

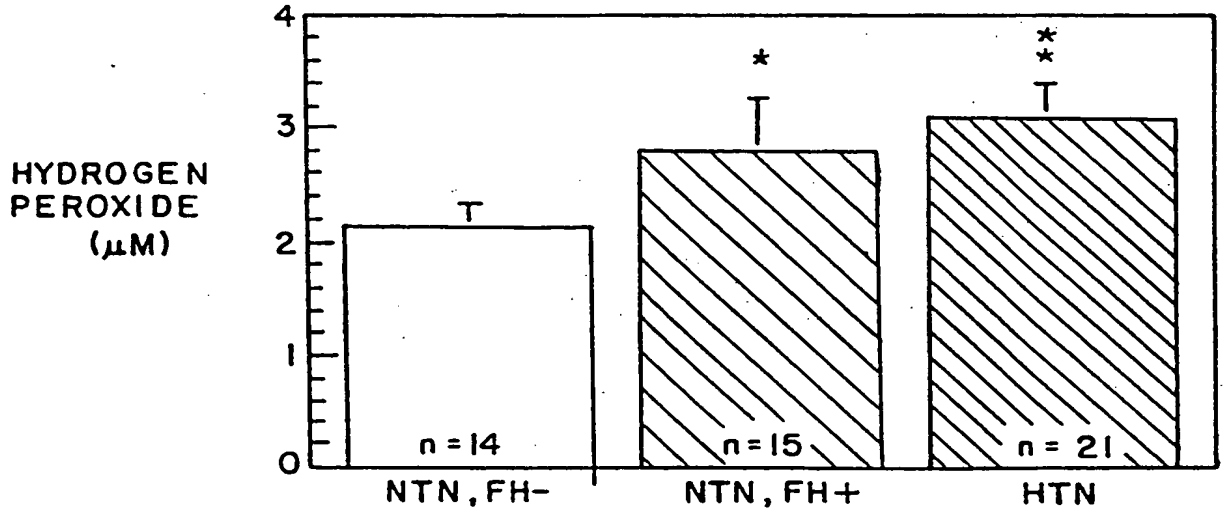
HYDROGEN PEROXIDE (μM)
4
3
2
1
0
*
**
n = 14
n = 15
n = 21
NTN, FH−
NTN, FH+
HTN

FIG. 4b

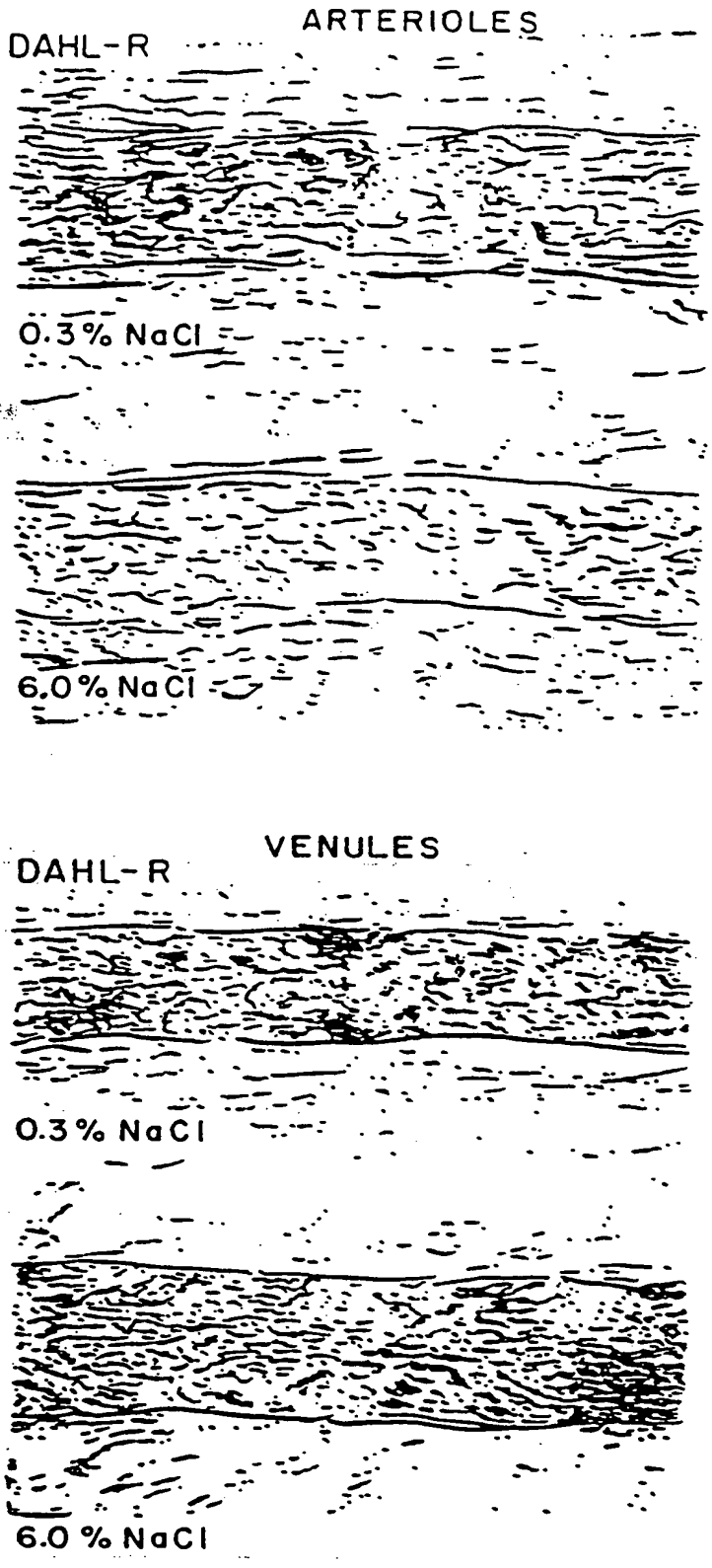

FIG. 6a

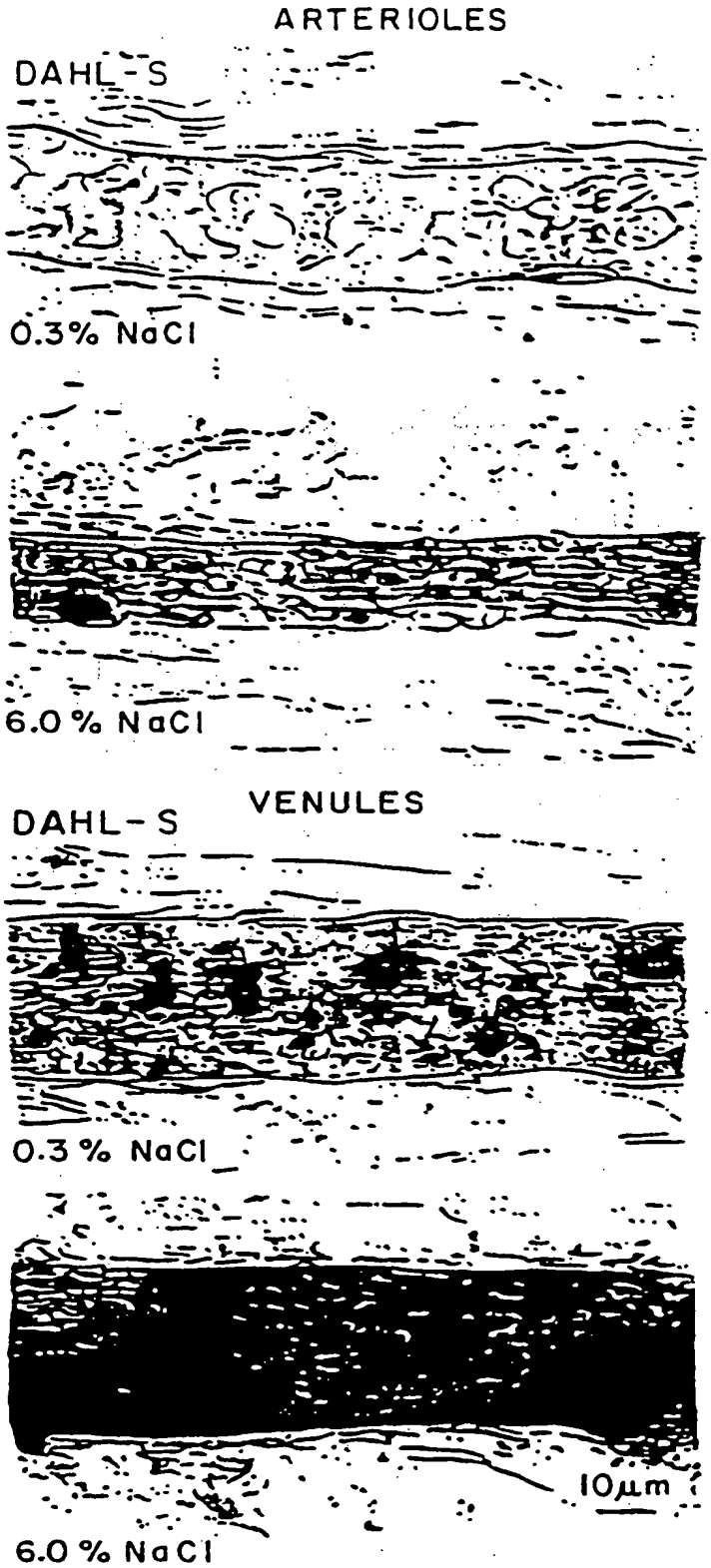
ARTERIOLES
DAHL-S
0.3% NaCl
6.0 % NaCl
VENULES
DAHL-S
0.3 % NaCl
10µm
6.0 % NaCl

FIG. 6b

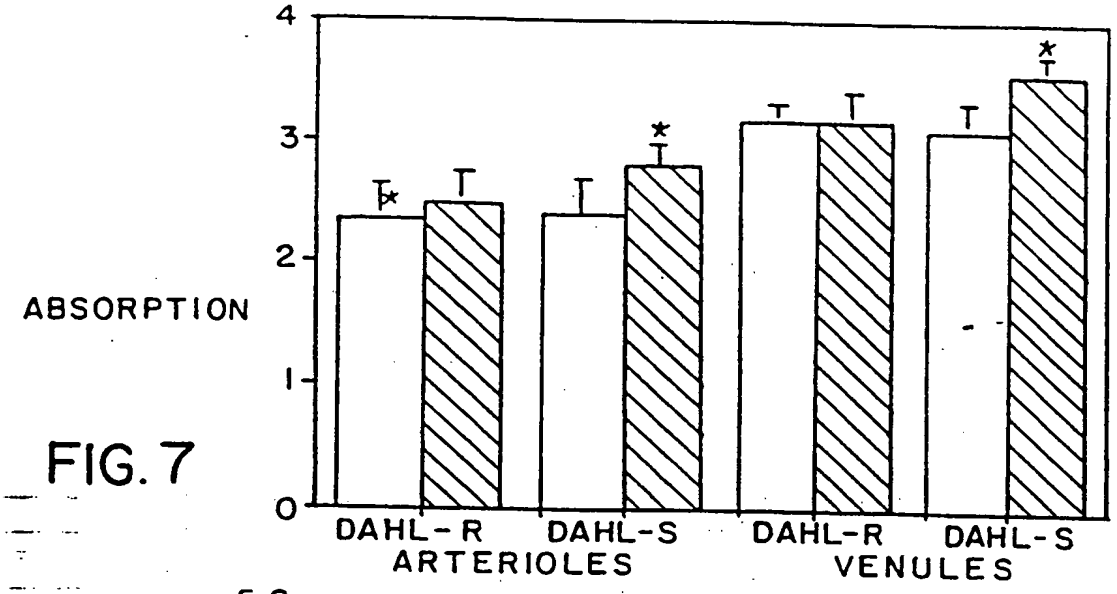

FIG. 7

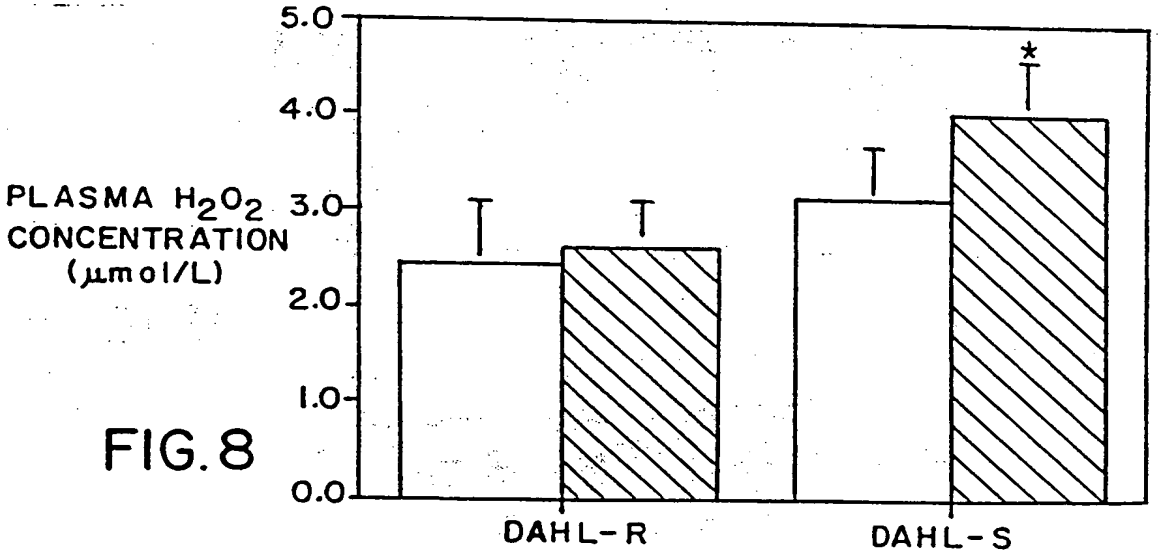

FIG. 8

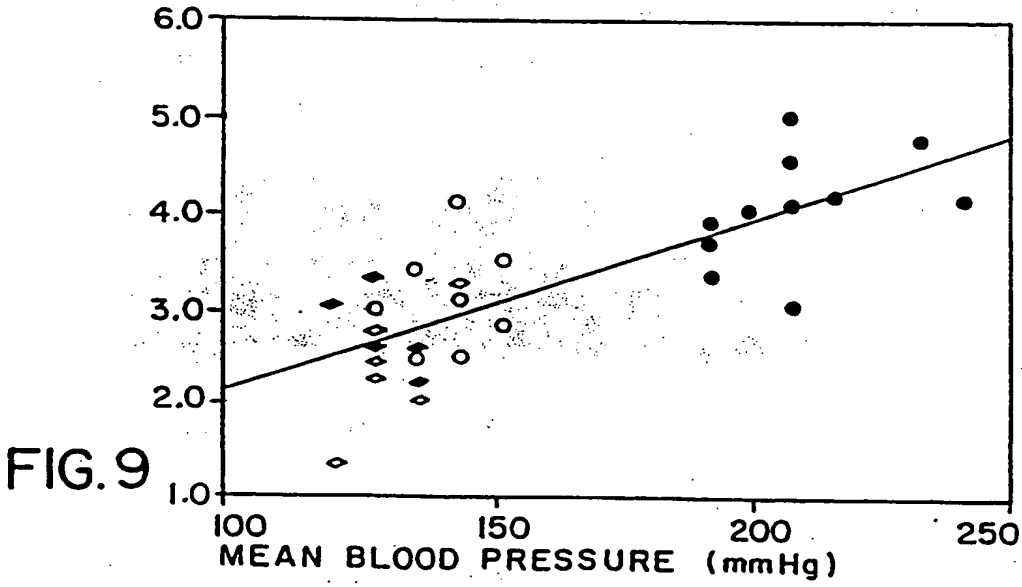

FIG. 9

INTERNATIONAL SEARCH REPORT

Int tional Application No
PCT/US 98/19013

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 G01N33/487 G01N33/49 G01N33/50 G01N27/26 C12Q1/00

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 G01N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	WO 94 02629 A (THE VICTORIA UNIVERSITY OF MANCHESTER) 3 February 1994 see the whole document ---	1-30
A	WO 90 02202 A (CETUS CORPORATION) 8 March 1990 see the whole document ---	1-30
A	DATABASE WPI Week 9427 Derwent Publications Ltd., London, GB; AN 94-224478 XP002090174 & SU 1 812 497 A (CRIMEA MED INST & UKR DOCTOR TRAINING INST), 25 December 1990 see abstract --- -/--	12-16

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

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Date of the actual completion of the international search	Date of mailing of the international search report
15 January 1999	25/01/1999

Name and mailing address of the ISA	Authorized officer
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C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

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A	US 5 320 725 A (GREGG) 14 June 1994 see column 4, line 30 - column 7, line 57; figures	1-30
A	US 4 340 448 A (SCHILLER ET AL.) 20 July 1982 see column 3, line 41 - column 6, line 50; figures 1-4	1-30

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